Glial Cell Line-Derived Neurotrophic Factor (GDNF) Receptor α-1 (GFRα1) Is Highly Selective for GDNF versus Artemin†

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Received April 15, 2004; Revised Manuscript Received October 12, 2004

ABSTRACT: To clarify whether glial cell line-derived neurotrophic factor (GDNF) receptor α-1 (GFRα1), the glycosylphosphatidylinositol (GPI)-linked coreceptor for GDNF, is also a functional coreceptor for artemin (ART), we have studied receptor binding, signaling, and neuronal survival. In cell-free binding studies, GFRα1−Ig displayed strong preferential binding to GDNF, though in the presence of soluble RET, weak binding to ART could also be detected. However, using GFRα1-transfected NB41A3 cells, ART showed no detectable competition against the binding of ^{125}I-labeled GDNF. Moreover, ART failed to induce phosphorylation of extracellular signal-related kinase (ERK) and Akt in these cells and was >10^4-fold less potent than GDNF in stimulating RET phosphorylation. When rat primary dorsal root ganglion (DRG) neurons were used, only the survival promoting activity of GDNF and not that of ART was blocked by an anti-GFRα1 antibody. These results indicate that although ART can interact weakly with soluble GFRα1 constructs under certain circumstances in vitro, in cell-based functional assays GFRα1 is at least 10 000-fold selective for GDNF over ART. The extremely high selectivity of GFRα1 for GDNF over ART and the low reactivity of ART for this receptor suggest that GFRα1 is not likely to be a functional coreceptor for ART in vivo.

Artemin (ART)[1] (*1*), also known as neublastin (*2*) and enovin (*3*), is a neurotrophic factor that is crucial for the proper development of the sympathetic nervous system (*4*, *5*). ART belongs to the glial cell line-derived neurotrophic factor (GDNF) family of ligands, which also includes GDNF, neurturin (NRTN), and persephin (PSPN) (*6*). These glycosylated, disulfide-linked homodimers mediate intracellular signaling via a receptor complex consisting of the transmembrane tyrosine kinase RET and a ligand-binding glycosylphosphatidylinositol-linked GDNF family receptor-α (GFRα) coreceptor. Although the four forms of GFRα (GFRα1−4) interact preferentially with GDNF, NRTN, ART, and PSPN, respectively, alternative interactions have been demonstrated in vitro (*7*−*11*). However, in the case of ART, its ability to interact with GFRα1, the preferred coreceptor for GDNF, is unresolved. Scott and Ibanez (*12*) showed that ^{125}I-ART was able to bind weakly to cells transfected with GFRα1. In addition, Baloh et al. (*1*) found that GFRα1 mediated ART signaling on transfected cells expressing RET and GFRα1. In contrast, Rakowicz et al. (*13*) reported that GDNF but not ART could signal through GFRα1 in motor neuron survival assays. It therefore remains unclear to what extent the reported interactions of ART with GFRα1 can induce RET phosphorylation and downstream signaling events with functional cellular consequences.

Expression of GFRα3, the preferred coreceptor for ART, is confined mainly to a subpopulation of developing and adult peripheral neurons (*14*−*19*), which are believed to be the primary site of ART action (*1*, *20*). In contrast, GFRα1 is much more widely distributed in both the peripheral and central nervous systems (*21*, *22*). Moreover, the phenotype of the GFRα1-deficient mouse is much more severe than that of the GFRα3-deficient mouse. While the GFRα1-deficient mouse has deficits in the kidneys, the enteric nervous system and spinal motor and sensory neurons and dies soon after birth (*23*, *24*), the GFRα3-deficient mouse is viable but has abnormalities in the migration and axonal projection pattern of sympathetic neurons (*4*, *5*). Thus, both the expression pattern of GFRα3 and the functional consequences of disrupting the GFRα3 gene indicate that the GFRα3 pathway has a more restricted role than the GFRα1 pathway during embryonic development. Consequently, if ART can also signal through GFRα1, then its range of

† The study was supported in part by the Quality of Life and Management of Living Resources Programme of the European Commission (Grant QIG3-CT-2002-01000).

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[1] Abbreviations: ART, artemin; DRG, dorsal root ganglion; DMEM, Dulbecco's modified Eagle's medium; EC_{50}, dose giving 50% maximal response; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-related kinase; FACS, fluorescence activated cell sorter; FBS, fetal bovine serum; GDNF, glial cell line-derived neurotrophic factor; GFRα, GDNF family receptor α chain; GFRα1−Ig, GFRα1 fused to the hinge, CH2, and CH3 portions of human IgG1; GFRα3−Ig, GFRα3 fused to the hinge, CH2, and CH3 portions of human IgG1; GPI, glycosylphosphatidylinositol; KIRA, kinase receptor activation; mAb, monoclonal antibody; NGF, nerve growth factor; NRTN, neurturin; pAb, polyclonal antibody; PSPN, persephin; RET−AP, RET extracellular domain fused to placental alkaline phosphatase; RET−Ig, RET extracellular domain fused to the hinge, CH2, and CH3 portions of human IgG1.

10.1021/bi049247p CCC: $30.25
Published on Web 00/00/0000 PAGE EST: 9.7

biological activities may be much broader than if it signals exclusively through GFRα3.

The phenotype of the ART-deficient mouse is very similar to that of the GFRα3-deficient mouse, suggesting that during development ART signaling is mediated exclusively by the GFRα3 coreceptor (*5*). However, this might not be the case in the adult state after injury or in a therapeutic setting where higher nonphysiological doses of ART may be administered. In a recent study, ART treatment resulted in the reversal of nerve injury-induced pain behavior and the normalization of a number of morphological and neurochemical features in an experimental model of neuropathic pain (*25*). The further development of ART as a therapeutic requires an understanding of its mechanism of action and a delineation of all receptors that can mediate its actions. In particular, whether ART can signal through only GFRα3 or through both GFRα3 and GFRα1 has implications for whether exogenous ART, administered as a drug, is likely to recapitulate some of the toxicity observed with GDNF (*26*–*28*).

In comparison to GDNF, the biochemistry of the interaction of ART with its receptors is relatively little studied. The aim of the current study is to clarify whether ART can interact with and signal through GFRα1 in vitro. We have examined the activity of ART, relative to that of GDNF, using assays measuring receptor binding, cell signaling, and survival of sensory neurons. We find that soluble forms of GFRα1 show very high selectivity for GDNF, though weak binding to ART can be detected under certain conditions. Binding of ART to GFRα1-transfected cells was at least 150-fold weaker than binding of GDNF. Moreover, in cell-based functional assays, ART showed little or no ability to signal via GFRα1, and ART-mediated survival of cultured sensory neurons was unaffected by an anti-GFRα1 antibody. Taken together, these studies indicate that GFRα1 is not likely to be a functional coreceptor for ART in vivo.

MATERIALS AND METHODS

Generation and Purification of Reagents

Rat artemin was prepared as described previously (*25*). Rat GDNF, human GFRα1−Ig, and murine RET−Ig were purchased from R&D Systems (Minneapolis, MN). Recombinant murine NGF was purchased from Promega, (Madison, WI). Rat GFRα1−Ig, rat RET−Ig, and RET−AP have been described previously (*10*). For ternary complex studies, rat GFRα1−Ig from Biogen Idec was used. For Biacore studies, rat or human GFRα1−Ig from R&D Systems was used. To generate GFRα3−Ig, a DNA fragment encoding the extracellular domain of murine GFRα3 (amino acids 1−397 of GenBank sequence AAC24354; murine GFRα3) was ligated to a fragment containing the Fc domain of human IgG1 and cloned into an expression vector to generate plasmid pGJ144. The plasmid was transfected into Chinese hamster ovary cells to generate a stable cell line producing the fusion protein, which was purified from conditioned medium using a protein A−Sepharose column (Amersham Biosciences, Piscataway, NJ).

Murine monoclonal antibody MAB560 was purchased from R&D Systems. Neutralizing sheep anti-NGF polyclonal antibody was purchased from Chemicon (Temecula, CA). Rabbit polyclonal antibody R1371, which recognizes human GFRα1, has been described previously (*29*). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Assay Measuring the Ability of ART or GDNF To Form a Ternary Complex with GFRα1−Ig or GFRα3−Ig and a RET−AP Fusion Protein.

Assays for ternary complex formation were performed as previously described (*10*) with minor modifications.

Surface Plasmon Resonance (Biacore) Measurements. GFRα1−Ig and GFRα3−Ig (60 μg/mL in 10 mM acetate, pH 6.0) were immobilized on different quadrants of a B1 sensor chip (Biacore AB, Uppsala, Sweden) through amine coupling, according to the manufacturer's instructions, to a surface density of approximately 5000 RU. All measurements were made using a Biacore 3000 (Biacore AB). To measure binding to the GFRα3−Ig or GFRα1−Ig on the chip surface, samples containing 75 nM ART or GDNF with or without 300 nM RET−Ig (*10*) were passed over the chip at a flow rate of 5 μL/min, using a running buffer containing 5 mM citrate, 150 mM NaCl, 3 mM $CaCl_2$, 0.1% bovine serum albumin, 0.005% p-20 (Biacore AB), pH 7.0. To measure the interaction between the various proteins in solution, an initial rates method was used (*30*). Under the conditions described (so-called "mass-transport-limited" conditions), the initial rate of binding is proportional to the concentration of ART in solution. Samples containing 75 nM ART plus 150 nM GFRα3− or GFRα1−Ig, with or without 300 nM RET−Ig, were allowed to equilibrate for 5 h at room temperature in running buffer. The equilibrated samples were then passed over the GFRα3−Ig- and GFRα1−Ig-derivatized chip surfaces at a flow rate of 5 μL/min for 12 min. The concentration of free ART present in each solution, and thus the extent to which ART had become bound by the other components in the solution, was determined from the initial velocity of binding with reference to a standard curve of 10−100 nM ART alone that was included in every experiment. Similar measurements were made using GDNF to measure its binding to GFRα1−Ig and to GFRα3−Ig with and without RET−Ig.

Generation and Characterization of Cell Lines Expressing GFRα1.

The murine neuroblastoma cell line NB41A3 (ATCC CCL 147), which expresses endogenous RET, was grown in DMEM (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS; JRH, Lenexa, KS), and 4 mM L-glutamine (BioWhittaker, Walkersville, MD). NB41A3 cells were transfected using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions with an expression plasmid containing the full-length sequence for human GFRα1, and stable transfectants were established in 250 μg/mL G418 (Invitrogen, Carlsbad, CA). Pools of high-expressing cells were selected by two rounds of preparative fluorescence-activated cell sorter (FACS) analysis, staining with rabbit anti-GFRα1 polyclonal antibody R1371 (*29*) using a MoFlo sorter (Cytomation, Fort Collins, CO). The resulting pooled culture was named NB41A3-hGFRα1. To generate the GFRα1 inducible cell line, NB41A3-hGFRα1-220, NB41A3 cells were transfected using LipofectAMINE PLUS (Invitrogen) with a plasmid containing the human

GFRα1 gene subcloned into the pJJ-EGFP vector, which allows regulation of GFRα1 and enhanced green fluorescent protein (EGFP) expression by doxycycline (*31*). Stable transfectants were selected using 100 μg/mL Zeocin (Invitrogen). High-expressing clones isolated with cloning cylinders were chosen on the basis of expression of EGFP and human GFRα1 mRNA (RT-PCR primers 5′-CCAGACCCG-GAGTTTCCTCTTT-3′ and 5′-AAGTACAGGGTCGCCAG-GAACA-3′, product of 182 bp), after induction with 1 μg/mL doxycycline for 24 h.

The rat pheochromocytoma cell line Neuroscreen-1 (Cellomics, Pittsburgh, PA), a PC12 subclone, which expresses endogenous RET, was grown in RPMI (BioWhittaker), 5% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 10% heat-inactivated donor horse serum (DHS; Hyclone), and 4 mM L-glutamine (BioWhittaker). Neuroscreen-1 cells were transfected using Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions with an expression plasmid containing the full-length sequence for rat GFRα1 (*10*).

Cell-Based Radioligand Binding Assays. NB41A3-hGFRα1 cells at 2.5×10^5 cells per well were pelleted in a V-bottom polypropylene plate (Nalge Nunc International, Rochester, NY). The supernatant was aspirated, and the cells were resuspended in reaction mixtures containing various concentrations of unlabeled rat GDNF or unlabeled rat ART and ^{125}I-GDNF (Amersham Biosciences) in a total volume of 50 μL of cell culture medium (DMEM, Invitrogen; 10% FBS, JRH, Lenexa, KS, 10 mM L-glutamine, pH 7.4). After incubation for 1 h at room temperature, the cells were pelleted through oil (one part phthalic acid plus two parts dibutyl phthalate). The cell-associated radioactivity was measured for each cell pellet using a γ counter (PerkinElmer, Wellesley, MA).

RET Phosphorylation ELISA. To assess the ability of ART and GDNF to activate RET, a modified kinase receptor activation (KIRA) ELISA (*32*) was used. NB41A3-hGFRα1 cells were seeded in 24-well plates (Corning, Corning, NY) at a density of 2×10^5 cells per well in growth medium. The next day, cells were stimulated in duplicate for 10 min with recombinant rat GDNF or recombinant rat ART in a volume of 250 μL. Cells were washed in PBS and lysed for 1 h on ice in 300 μL of a buffer consisting of 10 mM Tris, pH 8.0, 0.5% NP-40, 0.2% deoxycholic acid, 50 mM NaF, 0.1 mM Na_3VO_4, and 1 mM phenylmethanesulfonyl fluoride. In some cases, the lysates were frozen at −70 °C prior to performing the ELISA. For the ELISA, 96-well plates were coated with 5 μg/mL hamster anti-rat RET mAb AA.GE7.3 (*10*) in 50 mM carbonate buffer, pH 9.6, at 4 °C overnight followed by incubation in blocking buffer (Tris-buffered saline plus 0.05% Tween-20 containing 1% mouse serum and 3% bovine serum albumin) for 1 h at room temperature. A 270 μL aliquot of the lysate was added to each well, and the plate was incubated for 2 h at room temperature while shaking. The plate was washed in Tris-buffered saline containing 0.05% Tween-20, followed by incubation for 2 h with 2 μg/mL horseradish peroxidase-conjugated anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY) in blocking buffer. The plate was washed again before addition of 3,3′,5,5′-tetramethylbenzidine dihydrochloride. After the color reaction was stopped with 2 N H_2SO_4, absorbance values were read at 450 nm from wells treated with lysate or lysis buffer only, and the background-corrected signal was plotted as a function of the concentration of ligand used for stimulation.

Rat GFRα1 transiently transfected Neuroscreen-1 cells were assayed for activation by rat GDNF or rat ART in the KIRA ELISA as described above.

Intracellular Signaling Studies. NB41A3-hGFRα1-220 cells were seeded in 12-well plates at a density of 4×10^5 cells per well in growth medium. The next day, cells were stimulated with recombinant rat GDNF or recombinant rat ART for 10 min in serum-free DMEM and then harvested in 150 μL of sample buffer (2% SDS, 0.4 M Tris, pH 8.0, 10 mM DTT, and 0.25 mM Na_3VO_4). After incubation at 96 °C for 5 min, the samples were electrophoresed on 8−18% gradient SDS polyacrylamide gels (Amersham Pharmacia Biotech), followed by transfer to polyvinylidene fluoride membranes. Nonspecific binding sites were blocked by incubation in blocking buffer consisting of 5% skimmed milk and 0.1% Tween-20 in PBS. Signaling molecules were detected using anti-phospho-ERK mAb, (Clone E10; Cell Signaling Technology, Beverly, MA) at 1:2000 dilution or anti-phospho-Akt pAb (Ser-473; Cell Signaling Technology) at 1:1000 dilution, followed by the appropriate secondary horseradish peroxidase-linked antibody. Bands were detected by enhanced chemiluminescence using the ECL or the ECL Plus detection system (Amersham Biosciences).

P1 DRG Neuronal Survival Assays

Isolation and Culture of P1 DRG Cells. Dorsal root ganglia (DRG) from all spinal levels were removed from P1 (postnatal day 1) Sprague−Dawley rats (Charles River Laboratories, Cambridge, MA). Tissues were enzymatically dissociated in 125−250 U/mL type 1 collagenase (Worthington, Freehold, NJ) at 37 °C for 30 min. Samples were triturated with fire-polished Pasteur pipets and filtered though 70 μm sterile mesh to produce single cell suspensions. Cells were preplated on noncoated tissue-culture-ware dishes for 2 h to remove nonneuronal cells. Nonadherent cells were plated at 1.5×10^4 cells per well in 24-well tissue culture dishes that had been coated with poly(D-ornithine) (Life Technologies) and laminin (Collaborative Biomedical). Cells were cultured in UltraCULTURE serum-free medium (BioWhittaker) containing 2.5 μg/mL neutralizing sheep anti-NGF pAb. NGF-treated positive controls lacked the neutralizing anti-NGF pAb. The following factors were suspended in UltraCULTURE and added 1−2 h after plating: 50 ng/mL GDNF, 50 ng/mL recombinant murine NGF or 50 ng/mL ART with or without 10 μg/mL mouse anti-GFRα1 mAb MAB560 or 5 μg/mL rabbit anti-GFRα1 pAb R1371. Cultures were fed every second day by replacing the medium and growth factors.

Immunocytochemistry. After 7 days in culture, cells were fixed in 4% formaldehyde in PBS for 10 min at room temperature. Cells were preblocked in 4% goat serum and 0.1% Nonidet P 40 substitute (Sigma) for 30 min at room temperature and then incubated overnight at 4 °C with mouse anti-βIII tubulin (Sigma) at 1:100 dilution. After being rinsed in preblock solution, the cultures were incubated for 1 h at room temperature with Alexa-dye 584-conjugated anti-murine Ig antibody at 1:400 dilution (Molecular Probes, Eugene, OR). Following a final rinse in preblock solution,

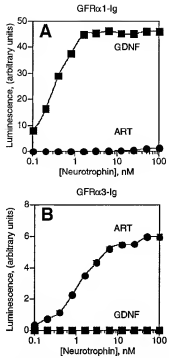

FIGURE 1: Ability of ART or GDNF to form a ternary complex with GFRα1−Ig and RET−AP. Anti-human Fc-coated microtiter plates were treated with the stated concentration of rat ART or GDNF to which was added a solution containing fixed concentrations of rat GFRα1−Ig or murine GFRα3−Ig and rat RET−AP conditioned medium. Plates were incubated for 1 h, and ternary complex formation was quantified by measuring bound RET−AP using the chemiluminescence substrate CSPD as described by Sanicola et al. (*10*). Panel A shows GFRα1−Ig incubated with ART (●) or GDNF (■) and RET−AP. Panel B shows GFRα3−Ig incubated with ART (●) or GDNF (■) and RET−AP. Error bars indicate the spread between duplicate measurements and are generally contained within the data symbols. The data shown are representative of three independent experiments.

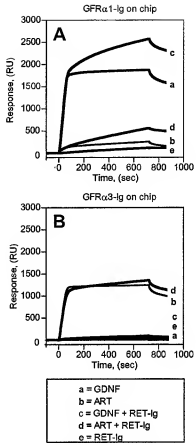

FIGURE 2: Ability of ART or GDNF to bind to bind to GFRα1−Ig immobilized on a Biacore chip surface. Rat GFRα1−Ig (A) or murine GFRα3−Ig (B) were immobilized to approximately equivalent densities on separate quadrants of a Biacore B1 chip as described in Materials and Methods. Solutions containing rat ART (75 nM), rat GDNF (75 nM), or rat RET−Ig (300 nM), alone or in combination, were passed over the chips as indicated: (a) GDNF alone; (b) ART alone; (c) GDNF + RET−Ig; (d) ART + RET−Ig; (e) RET−Ig alone. The data shown are representative of three independent experiments.

cells from a strip through the middle of each well were counted using a fluorescence microscope. All βIII-tubulin positive cells were scored as neurons, and survival was determined by the number of neurons counted per well. All antibodies were diluted in preblock solution.

Statistics. Data for neuronal survival assays were analyzed using JMP4 software (version 4.0.4 for Windows, SAS Institute Inc., Cary, NC). Significance was assessed using one-way ANOVA with Dunnett's post-hoc test.

RESULTS

Ability of ART or GDNF To Form a Ternary Complex with RET and GFRα1. The ability of ART or GDNF to form complexes with GFRα1 and RET was evaluated in a cell-free "ternary complex" ELISA. This ELISA measures the ability of ART or GDNF to mediate binding of a RET−alkaline phosphatase fusion protein (RET−AP) to assay plates coated with GFRα1−Ig. Figure 1A shows that GDNF induced formation of a ternary complex with RET−AP and GFRα1−Ig in a dose-dependent manner with half-maximal binding occurring at about 0.3 nM GDNF. Under the same conditions, ART appeared to show very little ternary complex formation, even at a concentration of 100 nM (Figure 1A). Although a weak signal was seen at concentrations of ART above 10 nM, binding was at least 100-fold weaker than that for GDNF. As a control, a similar experiment was performed using assay plates coated with GFRα3−Ig. Figure 1B shows that ART but not GDNF induced formation of a ternary complex with RET−AP and GFRα3−Ig with half-maximal binding occurring at ~1−2 nM ART. These results indicate that GFRα1−Ig and RET−AP form a stable ternary complex with GDNF but interact much more weakly with ART. A variation on this experiment, in which the GDNF or ART and the RET−AP were added to the plate in separate steps with a wash step between, gave a similar outcome (data not shown). This result suggests that the selectivity of GFRα1−Ig for GDNF over ART was principally manifested in the interaction with the GFRα1 alone.

Binding of ART or GDNF to GFRα1−Ig on a Biacore Chip Surface. To examine the interaction of ART with GFRα1−Ig in more detail, we performed binding experiments using Biacore. In these experiments, GFRα1−Ig was coupled to a Biacore chip surface. Solutions containing ART or GDNF, either alone or preincubated with RET−Ig, were passed over the chip and tested for their ability to interact with the GFRα1−Ig-derivitized surface. Figure 2A shows

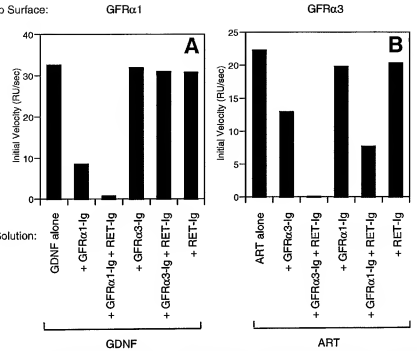

FIGURE 3: Ability of ART or GDNF to bind to GFRα1−Ig in solution, measured by Biacore. Rat GFRα1−Ig (A) or murine GFRα3−Ig (B) were immobilized to approximately equivalent densities on separate quadrants of a Biacore B1 chip. Rat ART (75 nM) or GDNF (75 nM) were preincubated with 150 nM GFRα1−Ig or GFRα3−Ig, either with or without 300 nM rat RET−Ig, and allowed to reach equilibrium. The preincubated solutions were passed over the Biacore chip surface. The initial rate of binding (V_i) measured over the first 50 s of each sensorgram is plotted for each sample as a measure of the fraction of unbound neurotrophin present in each solution. The components present in each equilibrated solution are indicated below each bar on the plot. The data shown are representative of three independent experiments.

that 75 nM GDNF (a) interacted strongly with the GFRα1−Ig surface, whereas 75 nM ART (b) showed no appreciable interaction with the immobilized GFRα1−Ig. When a mixture containing GDNF plus 300 nM RET−Ig (c) was passed over the chip, after the initial rapid binding of GDNF to the chip a second slower phase of binding was observed. This result suggests that the rapid binding of GDNF to the GFRα1−Ig chip surface is followed by the slower binding of RET−Ig to the resulting GDNF/GFRα1−Ig binary complex on the chip surface to form a GFRα1−Ig/GDNF/RET−Ig ternary complex. Interestingly, a mixture containing ART plus 300 nM RET−Ig (d) gave a low (relative to GDNF alone or GDNF plus RET−Ig) but reproducible level of binding to the immobilized GFRα1−Ig. This binding was slow and was not seen with either ART alone or Ret−Ig alone. This signal therefore indicates that while no stable binary complex between GFRα1−Ig and ART can be observed, the inclusion of a very high concentration of RET−Ig can induce formation of a ternary complex containing ART and RET−Ig bound to the immobilized GFRα1−Ig. Similar results were obtained when human GFRα1−Ig was used in place of rat GFRα1−Ig to generate the Biacore chip surface (data not shown). The relatively low signal observed for ART plus RET−Ig suggests that binding is weak, not only relative to GDNF plus RET−Ig but also compared to GDNF alone. Once again the reciprocal experiment was performed as a control. Figure 2B shows that, as expected, ART, either alone (b) or in the presence of RET−Ig (d), interacted strongly with the immobilized GFRα3−Ig, whereas GDNF showed no appreciable binding to the GFRα3−Ig chip surface either with or without RET−Ig (a and c). As was seen for GDNF plus RET−Ig binding to the GFRα1−Ig chip, ART binding to the GFRα3−Ig chip surface in the presence of RET−Ig results in biphasic kinetics as the rapid binding of ART is followed by the slower binding of RET−Ig to form a ternary complex on the chip surface. As expected, RET−Ig (e) does not interact with GFRα3−Ig in the absence of neurotrophin.

Binding of ART or GDNF to GFRα3−Ig or GFRα1−Ig in Solution. We additionally employed an alternative Biacore method to study the interactions between the proteins in solution, by preincubating ART or GDNF with soluble GFRα1−Ig before passage over the chip. Under the experimental conditions described in Materials and Methods, the initial velocity of binding of ART to a GFRα3−Ig chip surface or of GDNF to a GFRα1−Ig chip surface is proportional to the concentration of free neurotrophin in solution (data not shown). Preincubation of ART with excess soluble GFRα3−Ig rendered the resulting mixture unable to bind to the GFRα3−Ig chip surface indicating, as expected, that this surface recognizes only free ART (Figure 3 and data not shown). Similarly, preincubation of GDNF with excess GFRα1−Ig in solution blocked the binding of GDNF to a GFRα1−Ig-derivitized Biacore chip surface. Thus, preincubating ART or GDNF with soluble receptor proteins and analyzing the resulting mixtures on the appropriate Biacore chip surface (GFRα3−Ig for ART and GFRα1−Ig for GDNF) provides a measure of bound versus free ART or GDNF in the preincubated mixtures. Because the initial velocity of binding is proportional to free ART or GDNF, the concentration of free neurotrophins—and thus the extent of binding in the preincubated solutions—can be

measured with precision. In these experiments, the contact time of the solution with the chip surface is very short (~250 ms), so re-equilibration of the reaction mixtures is likely to be negligible. This method therefore provides an essentially instantaneous snapshot of the ratio of bound versus free neurotrophin that is present in the pre-equilibrated solutions before they encounter the Biacore chip surface.

Figure 3A shows that 75 nM GDNF preincubated to equilibrium with 150 nM GFRα1−Ig resulted in a substantially reduced initial velocity compared to GDNF alone indicating that, under these conditions, ~70% of the GDNF was bound in a complex by the soluble GFRα1−Ig. Inclusion of 300 nM RET−Ig in the solution resulted in substantially all of the GDNF becoming bound, indicating the formation of a highly stable ternary complex between GDNF, GFRα1−Ig, and RET−Ig in solution. These results are consistent with those in Figure 2A and also with the results of the ternary complex ELISA (Figure 1A), which showed the formation of a stable ternary complex between GDNF, GFRα1−Ig, and RET−AP. In contrast, preincubation of GDNF with GFRα3−Ig plus or minus 300 nM RET−Ig gave no significant reduction in the initial velocity of binding, indicating that no significant fraction of the GDNF present in the preincubated solutions was bound. This finding is consistent with the results from Figures 1B and 2B, which similarly showed that GDNF does not bind detectably to GFRα3−Ig even in the presence of soluble RET. Figure 3B shows that preincubation of ART and GFRα3−Ig induces a significant level of complex formation and that inclusion of RET−Ig in the mixture results in ~100% of the ART becoming bound in a ternary complex with GFRα3−Ig and RET−Ig in solution, consistent with the results in Figures 1B and 2B. Importantly, Figure 3B shows that preincubating ART and GFRα1−Ig in solution results in no significant complex formation. However, in the presence of 300 nM RET−Ig, a significant fraction of the ART became bound in a ternary complex with GFRα1−Ig and RET−Ig in solution. These results confirm that the direct binding of ART alone to GFRα1−Ig cannot be detected under the fairly stringent conditions used, and thus if it occurs it must be extremely weak compared to the binding of GDNF to GFRα1−Ig or ART to GFRα3−Ig. Nevertheless, in the presence of a high concentration of soluble RET−Ig, a stable ternary complex between ART, GFRα1−Ig, and RET−Ig can form. Figures 1−3 show, however, that this ternary complex is weak compared to the corresponding ternary complex formed between GDNF, GFRα1−Ig, and RET. Figure 3B in particular shows that, even in the highly stabilizing presence of a high concentration of RET−Ig, ART is bound only about as strongly as GDNF binds to GFRα1−Ig in the absence of RET−Ig and significantly less strongly than GDNF binds to GFRα1−Ig or ART binds to GFRα3−Ig in the presence of RET−Ig.

Binding of ART and GDNF to Cell Surface GFRα1. To test whether the binding results obtained using soluble GFRα1−Ig can be extrapolated to GFRα1 expressed on the cell surface, the activity of GDNF and ART were compared in a variety of cell-based binding and functional assays. We were particularly interested in whether RET expressed on cells was able to mediate binding of ART to GFRα1 and, if so, whether the resulting complex induced RET signaling and downstream functional responses. As a first step, a radioligand binding assay was developed using NB41A3-hGFRα1 cells, which endogenously express murine RET and were stably transfected with GPI-linked human GFRα1. Functional mapping of receptor binding domains of GDNF family ligands has shown that the structural determinants for their binding to GFRα1 are similar (*8*). It is therefore likely that, if ART were able to bind to GFRα1, its binding site would overlap with that of GDNF and thus binding of ART and GDNF would be competitive. Figure 4A shows that the binding of ^{125}I-GDNF to the GFRα1-expressing cells was saturable with a K_D of ~2 nM and that no such saturable binding was detected using untransfected parental NB41A3

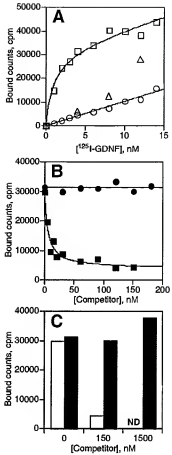

FIGURE 4: Ability of GDNF or ART to bind to GFRα1 expressing cells: (A) rat ^{125}I-GDNF, at the specified concentrations, was incubated with parental NB41A3 cells (△) or with NB41A3-hGFRα1 cells with (○) or without (□) 1 μM unlabeled rat GDNF; (B) NB41A3-hGFRα1 cells were incubated with 5 nM rat^{125}I-GDNF plus the indicated concentration of either unlabeled rat ART (●) or unlabeled GDNF (■); (C) NB41A3-hGFRα1 cells were incubated with 8 nM rat ^{125}I-GDNF plus the indicated concentration of unlabeled rat ART (black bars) or unlabeled rat GDNF (white bars). All incubations were for 60 min at room temperature. In panel C, competition with 1500 nM unlabeled GDNF was not done (ND). Bound counts were separated from unbound by pelleting cells through oil as described in Materials and Methods. Data in panel A were fitted to a straight line (○) or to the equation for saturable plus nonspecific binding (□): cpm = {(cpm$_{max}$[^{125}I-GDNF])/(K_D + [^{125}I-GDNF])} + s[^{125}I-GDNF], where s is the slope of the linear fit for nonspecific binding only. Similar results were obtained in three independent experiments.

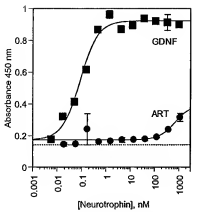

FIGURE 5: Ability of ART or GDNF to stimulate RET phosphorylation in NB41A3-hGFRα1 cells. Murine NB41A3 cells stably transfected with human GFRα1 were examined for RET phosphorylation after stimulation with the indicated concentration of rat ART (●) or rat GDNF (■) using a kinase receptor activation (KIRA) ELISA bioassay as described in Materials and Methods. The data shown are representative of six independent experiments (except that, unlike the example shown, three of the replicates showed absolutely no ART-dependent signal up 400 nM or 1 μM ART). Data were fitted using a standard four-parameter equation ($y = \{(a - d)/(1 + (x/c)^b)\} + d$). The dashed line represents background phosphorylation of cells treated identically but without neurotrophin.

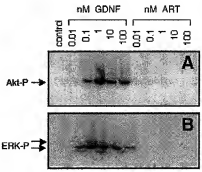

FIGURE 6: Ability of ART or GDNF to stimulate downstream signaling of NB41A3-hGFRα1-220 cells. NB41A3-hGFRα1-220 cells were examined by Western blot analysis after stimulation with the indicated concentration of rat ART or rat GDNF. Cell lysates were assayed by immunoblotting with either an anti-phospho-Akt (A) or an anti-phospho-ERK (B) antibody and an appropriate secondary antibody as described in Materials and Methods. The results shown are representative of three independent experiments.

cells. Figure 4B shows that binding of 5 nM ^{125}I-GDNF was inhibited by unlabeled GDNF with an IC_{50} of ~10 nM, consistent with the expected K_D of ~2 nM after correction for competition by the labeled GDNF. In contrast, unlabeled ART (up to 180 nM, Figure 4B) did not compete against ^{125}I-GDNF binding to NB41A3-hGFRα1 cells. In similar experiments, ART concentrations of up to 1.5 μM were tested, and no inhibition was observed (Figure 4C), indicating that binding of ART to the cell-surface GFRα1 is weak or absent ($K_D \geq 300$ nM). In the Biacore binding experiments shown in Figures 2 and 3, it appeared that a weak interaction between ART and GFRα1 could be detected in the presence of RET−Ig. We therefore tested to see whether including soluble RET−Ig might similarly mediate the binding of ART to the GFRα1-expressing cells. Inclusion of 300 nM RET−Ig in similar competition binding assays did not affect the inability of ART at concentrations up to 1 μM to compete against 5 nM ^{125}I-GDNF (data not shown). These results indicate that cell-surface bound GFRα1 is selective for binding GDNF over ART by a factor of > 150-fold with no interaction of ART with GFRα1 being detected in these experiments.

RET Phosphorylation and Downstream Signaling in NB41A3 Cells. Activation of RET induced by ART or GDNF was examined in NB41A3-hGFRα1 cells using a kinase receptor activation (KIRA) ELISA bioassay, similar to that developed for a different receptor by Sadick et al. (*32*). On NB41A3-hGFRα1 cells GDNF induced dose-dependent phosphorylation of RET with an EC_{50} of 0.1 nM, whereas ART elicited no detectable RET phosphorylation at concentrations up to 100 nM (Figure 5). Three of the six times that this experiment was performed (including the example shown in Figure 5), a very weak response was seen at concentrations greater than 100 nM ART. However, in all cases the potency of this response was at least 10 000-fold lower than that for GDNF. Consistent with these findings, concentrations of GDNF as low as 0.01−0.1 nM induced phosphorylation of the downstream signaling molecules Akt and ERK in NB41A3-hGFRα1-220 cells, whereas up to 100 nM ART had no detectable effect (Figure 6). No RET phosphorylation or downstream signaling could be detected in parental (untransfected) cells treated with GDNF or ART (data not shown). To establish that the low response of the NB41A3-hGFRα1 cells to ART was not because the receptor components in this experiment were not all from the same species, the KIRA ELISA was repeated using all rat components (data not shown) by transiently transfecting Neuroscreen-1 cells with rat GFRα1. Although these cells gave a weaker signal, the results showed the same high selectivity for stimulation by GDNF versus ART that was seen using the NB41A3 cells.

GDNF, but not ART, Promotes the Survival of Dorsal Root Ganglion Sensory Neurons through GFRα1. Previous studies have demonstrated that, in addition to RET, both GFRα1 and GFRα3 are expressed in neurons from dorsal root ganglia (DRG neurons) (*20*, *33*). To investigate whether any measurable portion of the survival effects of ART on cultured sensory neurons is mediated through GFRα1, we examined the survival promoting effects of fixed doses (50 ng/mL) of ART or GDNF in the presence and absence of a neutralizing anti-rat GFRα1 antibody, MAB560. In the absence of MAB560, both ART and GDNF caused an increase in the number of βIII-tubulin-positive neurons equivalent to that observed in NGF-treated cultures (Figure 7). However, in the presence of 10 μg/mL of MAB560, only NGF and ART treatment increased the number of βIII-positive neurons above control. In the cultures treated with GDNF and MAB560 there was an 80% decrease in neuronal survival as compared to GDNF alone, and the neuronal survival observed in these cultures was not above background. In contrast, MAB560 did not alter the survival-promoting effect of ART, indicating that GFRα1 does not mediate this effect. A similar effect was also seen using an anti-ratGFRα1 polyclonal antibody, R1371, which, like MAB560, inhibited the survival effect of GDNF but not ART (data not shown).

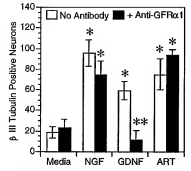

FIGURE 7: GFRα1-dependent survival of rat DRG sensory neurons. P1 rat DRG neurons were cultured in serum-free medium. Samples were treated with 50 ng/mL murine NGF, 50 ng/mL rat GDNF, or 50 ng/mL rat ART in the presence or absence of 10 μg/mL blocking anti-human GFRα1 antibody MAB560. Data represent the mean ± standard error of two independent experiments, each with three samples. Single asterisk denotes significantly different from media alone ($P < 0.05$). Double asterisk denotes significantly different from GDNF alone ($P < 0.05$).

DISCUSSION

Neurotrophic factors are important regulators of the development and homeostasis of many cells and tissues within the nervous system (*34*). Efforts to develop neurotrophic factors as therapeutics have sometimes encountered problems due to unexpected side effects resulting from a broader receptor distribution than initially appreciated (*35*). Since ART may have potential therapeutic utility in neuropathic pain, it is essential to identify all receptors that mediate its actions. It has been established that the preferred coreceptor for ART is GFRα3. However, there are conflicting reports concerning whether ART can cross-react with GFRα1, the preferred coreceptor for GDNF. While the similarity in phenotypes between the ART and GFRα3 knock-out mice indicates that during development ART signals only through GFRα3, this may not be true in the adult after injury. Furthermore, ART might cross-react with GFRα1 when administered at higher than physiological doses and therefore might have the same undesired side effects as GDNF when used as a drug.

In this study we have characterized the ability of ART to interact with GFRα1, the preferred coreceptor for GDNF. Cell-free binding assays using GFRα1−Ig, either captured on an ELISA plate, immobilized on a Biacore chip surface, or in solution, showed that this molecule possesses a very high selectivity for binding to GDNF over ART. No binding between ART and GFRα1−Ig could be detected in the absence of added soluble RET. In the presence of a high concentration of soluble RET, however, a weak complex between ART, soluble RET, and GFRα1−Ig could be detected in all three experimental formats that used this soluble GFRα1 construct. These cell-free experiments indicate that, at least under forcing conditions, ART can interact with GFRα1 and RET. However, it is not trivial to extrapolate from these solution-phase binding results to the cell. There is no established theoretical basis for predicting, for any given levels of GFRα1 and RET expression on a cell, whether the interaction will or will not occur. Therefore, to establish the functional relevance of the weak binding seen between ART and GFRα1−Ig, we directly tested the ability of ART to interact with and signal through GFRα1 using a number of different cell-based assays.

Competition radioligand binding measurements using NB41A3 cells stably expressing GPI-linked GFRα1 failed to detect any binding of ART, even in the presence of 300 nM soluble RET−Ig. These results established that binding of ART to cell-surface GFRα1, if it occurs at all, is at least 150-fold weaker than the binding of GDNF under these conditions. Similarly, in the signaling studies, the NB41A3-hGFRα1 cells displayed >10 000-fold selectivity for GDNF over ART in a KIRA ELISA measuring RET phosphorylation, and no phosphorylation of Akt or ERK could be detected upon treatment with ART at concentrations up to 1000−10 000-fold higher than those required for activity of GDNF. Although in the KIRA experiments a low level of response was sometimes seen at ART concentrations >100 nM, this is much higher than the concentrations of ART detected after an efficacious dosing regimen in the rat spinal nerve ligation model described by Gardell et al. (*25*) (data not shown). The KIRA ELISA was also performed using all rat components and gave a similar result. This result demonstrates that the low ability of ART to signal via GFRα1 is an intrinsic property of the receptor and is not due to a difference in the binding affinities of rat ART for human GFRα1/mouse RET versus rat GFRα1/rat RET. Experiments on primary neuronal cells were entirely consistent with the results obtained with the transfected NB41A3-hGFRα1 cells in showing that the signaling and downstream functions of ART are not mediated by GFRα1 to any significant extent.

Published studies have given conflicting data concerning whether ART is specific for GFRα3 or whether it can signal via GFRα1. For example, Baloh et al. (*1*) showed that GFRα3−Ig but not GFRα1−Ig could bind to ART-coated ELISA plates but that ART was able activate a reporter gene in MG87 fibroblasts transiently expressing GFRα1. More recent results showed, however, that GDNF but not ART induced survival of GFRα1-expressing motor neurons (*13*). In the present study, ART showed little or no ability to mediate a GFRα1-dependent functional response on either GFRα1 transfected or untransfected cells expressing endogenous RET. A possible explanation for differences between the behavior of the ART used in the present study and that seen in some previous studies might involve differences in the purification protocols or formulation buffer used for ART, leading to different levels of aggregated protein present as low-level contaminants. In other experiments, we have observed that aggregated forms of ART can show anomalous binding and signaling behavior (data not shown). The preparations used in the present study were purified and formulated such that aggregate formation was minimized. Another possibility is that the ability of ART to mediate GFRα1-dependent functional responses may differ for cells that express endogenous GFRα1 and RET versus transfected cells (*1*, *8*, *13*). Alternatively, it is possible that the ability of a low level of ART/GFRα1 complex to mediate function depends on the cell type used in the experiments.

It is very difficult to say how the expression level of RET on the cells used in our experiments might compare to the concentration of 300 nM RET−Ig that was required to induce some level of ternary complex formation in solution. However, we found that the level of RET expression on

NB41A3 cells is high compared to all other neuronal or nonneuronal RET-expressing cell lines that we tested, as measured by IP/Western blot or by flow cytometry (data not shown). Thus, the >1000-fold selectivity displayed by these cells for stimulation by GDNF versus ART is probably representative of other RET-expressing cells. In particular, the antibody blocking experiment using DRG neurons, described in Figure 7, indicates that GFRα1 does not mediate any significant response to ART in this highly relevant primary cell type.

We conclude that, despite the ability of ART to form a weak ternary complex with GFRα1−Ig and RET−Ig in solution, there is little or no functional cross-reactivity between ART and GFRα1 on cells. Most likely, the interaction between ART and GFRα1 is so weak that, at the concentrations of GFRα1 and RET present on cells, no significant complex formation occurs. The alternative possibility that ART does indeed induce the formation of a ternary complex with GFRα1 and RET in some of our cell-based experiments but that the resulting complex is not competent to mediate RET phosphorylation and downstream signaling cannot formally be ruled out, though such an explanation would not alter our basic conclusion. While the crossover in activities between GDNF and NRTN is clear (*36*, *37*), our results indicate that ART, like PSPN, appears to signal via a single GFRα family member (*38*). In this respect, the GFRα family of receptors resembles certain other receptor families, such as the TNF receptor superfamily (*39*), the class I cytokine receptors (*40*), and the EGF receptor family (*41*), in that certain ligands and receptors from the family are able to functionally interact with two or more partners, whereas other family members manifest a strict specificity.

ART has been proposed as a systemic treatment for neuropathic pain (*25*). Because administration of GDNF to rodents (*26*, *28*, *42*) has been associated with side effects such as weight loss, most likely mediated by interaction with GFRα1, our findings also have implications for the pharmaceutical development of recombinant ART. The high selectivity of ART for GFRα3 coupled with the restrictive expression of GFRα3 to a subpopulation of sensory neurons is likely to give ART a very specific therapeutic profile. In particular, the relative inability of ART to signal through GFRα1 suggests that recombinant ART will avoid the presumably GFRα1-dependent side effects observed with GDNF.

ACKNOWLEDGMENT

We express appreciation to Konrad Miatkowski and Joseph Amatucci for assistance in producing the murine GFRα3−Ig fusion protein, Michele McAuliffe, Brittney Coleman, and Christopher Tonkin for DNA sequencing, Raymond Boynton, Azita Kaffashan, and Carmen Young for protein sequencing and characterization, Akos Szilvasi for flow cytometry analysis, Gary Jaworski for plasmid pGJ144, the Biogen Idec Media Preparation Group, and Hanne Fosmark, NsGene A/S, for providing excellent technical assistance.

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BI049247P